

# Circulation Research

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# Exacerbation of Cerebral Injury in Mice That Express the P-Selectin Gene

## Identification of P-Selectin Blockade as a New Target for the Treatment of Stroke

E.S. Connolly, Jr, C.J. Winfree, C.J. Prestigiacomo, S.C. Kim, T.F. Choudhri, B.L. Hoh, Y. Naka, R.A. Solomon, D.J. Pinsky

**Abstract** There is currently a stark therapeutic void in the treatment of evolving stroke. Although P-selectin is rapidly expressed by hypoxic endothelial cells in vitro, the functional significance of P-selectin expression in stroke remains unexplored. In order to identify the pathophysiological consequences of P-selectin expression and to identify P-selectin blockade as a potential new approach for the treatment of stroke, experiments were performed using a murine model of focal cerebral ischemia and reperfusion. Early P-selectin expression in the postischemic cerebral cortex was demonstrated by the specific accumulation of radiolabeled anti-murine P-selectin IgG, with the increased P-selectin expression localized to the ipsilateral cerebral microvascular endothelial cells by immunohistochemistry. In experiments designed to test the functional significance of increased P-selectin expression in stroke, neutrophil accumulation in the ischemic cortex of mice expressing the P-selectin gene (PS +/+) was demonstrated to be significantly greater than that in homozygous P-selectin-null

mice (PS -/-). Reduced neutrophil influx was accompanied by greater postischemic cerebral reflow (measured by laser Doppler) in the PS -/- mice. In addition, PS -/- mice demonstrated smaller infarct volumes (5-fold reduction,  $P < .05$ ) and improved survival compared with PS +/+ mice (88% versus 44%,  $P < .05$ ). Functional blockade of P-selectin in PS +/+ mice using a monoclonal antibody directed against murine P-selectin also improved early reflow and stroke outcome compared with control mice, with reduced cerebral infarction volumes noted even when the blocking antibody was administered after occlusion of the middle cerebral artery. These data are the first to demonstrate a pathophysiological role for P-selectin in stroke and suggest that P-selectin blockade may represent a new therapeutic target in the treatment of stroke. (*Circ Res.* 1997;81:304-310.)

**Key Words** • P-selectin • stroke • adhesion molecule • transgenic mouse • neutrophil

Ischemic stroke constitutes the third leading cause of death in the United States today.<sup>1</sup> Until very recently, there has been no direct treatment to reduce cerebral tissue damage in evolving stroke. Although the NINDS<sup>2</sup> and ECASS<sup>3</sup> rt-PA<sup>1</sup> acute stroke studies have suggested that there are potential therapeutic benefits of early reperfusion,<sup>4</sup> the increased mortality observed after streptokinase treatment of acute ischemic stroke<sup>5</sup> highlights the sobering fact that there is at the present time no clearly effective treatment for evolving stroke. This void in the present medical armamentarium for the treatment of stroke has led to a number of innovative approaches,<sup>6</sup> yet, other than rt-PA, none has reached the clinical realm. To identify a potential safe and efficacious treatment for evolving stroke, we have focused on the deleterious role of recruited neutrophils. Recent work in a murine model of reperfused stroke has demonstrated that depletion of neutrophils (PMNs) before stroke minimizes cerebral tissue injury and improves functional outcome<sup>7</sup>; mice that lack the specific

cell adhesion molecule, ICAM-1, are similarly protected.<sup>7</sup> P-Selectin, a molecule that can be rapidly translocated to the hypoxic endothelial surface from preformed storage sites,<sup>8</sup> is an important early mediator of neutrophil rolling,<sup>9</sup> which facilitates ICAM-1-mediated neutrophil arrest. Although P-selectin is expressed in primate stroke,<sup>10</sup> the functional significance of P-selectin expression in stroke remains unknown.

To explore the pathophysiological role of P-selectin in stroke, we used a murine model of focal cerebral ischemia and reperfusion<sup>11</sup> involving both wild-type mice and mice that were homozygous null for the P-selectin gene<sup>9</sup> and a strategy of administering a functionally blocking P-selectin antibody. In these studies, we confirm not only that P-selectin expression after MCAO is associated with reduced cerebral reflow after reperfusion and a worse outcome after stroke, but that P-selectin blockade confers a significant degree of postischemic cerebral protection. These studies represent the first demonstration of the pathophysiological role of P-selectin expression in stroke and suggest the exciting possibility that anti-P-selectin strategies may prove useful in the treatment of reperfused stroke.

### Materials and Methods

#### Mice

Experiments were performed with transgenic, P-selectin-deficient mice (generously provided by Dr Denisa Wagner),

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**Selected Abbreviations and Acronyms**

ICAM-1	= intercellular adhesion molecule-1
MCAO	= middle cerebral artery occlusion
PMN	= polymorphonuclear leukocyte
PS $-/-$ mice	= P-selectin-null mice
PS $+/+$ mice	= P-selectin wild-type mice
rt-PA	= recombinant tissue plasminogen activator
TTC	= 2,3,5-triphenyl-2H-tetrazolium chloride

created as previously reported<sup>9</sup> by gene targeting in J1 embryonic stem cells, injected into C57BL/6 blastocysts to obtain germline transmission, and backcrossed to obtain homozygous P-selectin-null mice (PS  $-/-$ ). Experiments were performed with PS  $-/-$  or wild-type (PS  $+/+$ ) cousin mice from the third generation of backcrossings with C57BL/6J mice. Animals were 7 to 12 weeks of age and weighed between 25 and 36 g at the time of the experiments. Because variations in cerebrovascular anatomy have been reported to result in differences in susceptibility to experimental stroke in mice,<sup>12</sup> India ink/carbon black staining was performed to visualize the vascular pattern of the circle of Willis in both in both PS  $-/-$  and PS  $+/+$  mice. These experiments demonstrated that there were no gross anatomic differences in the vascular pattern of the cerebral circulation.

**Transient MCAO**

Mice were anesthetized (0.3 mL of 10 mg/mL ketamine and 0.5 mg/mL xylazine IP) and positioned supine on a rectal temperature-controlled operating surface (Yellow Springs Instruments, Inc). Animal core temperature was maintained at  $37 \pm 1^\circ\text{C}$  during surgery and for 90 minutes after surgery. A midline neck incision was created to expose the right carotid sheath under the operating microscope ( $\times 16$  to  $\times 25$  zoom, Zeiss). The common carotid artery was isolated with a 4-0 silk, and the occipital, pterygopalatine, and external carotid arteries were each isolated and divided. MCAO was accomplished by advancing a 13-mm heat-blunted 5-0 nylon suture via the external carotid stump. After placement of the occluding suture, the external carotid artery stump was cauterized, and the wound was closed. After 45 minutes, the occluding suture was withdrawn to establish reperfusion. These procedures have been previously described in detail.<sup>11</sup>

**Measurement of Cerebral Cortical Blood Flow**

Transcranial measurements of cerebral blood flow were made using laser Doppler (Perimed, Inc), as previously described.<sup>13</sup> Using a 0.7-mm straight laser Doppler probe (model PF303, Perimed) and previously published landmarks (2 mm posterior to the bregma, 6 mm to each side of midline),<sup>11</sup> relative cerebral blood flow measurements were made as indicated: immediately after anesthesia, 1 and 10 minutes after occlusion of the middle cerebral artery, and after 30 minutes, 300 minutes, and 22 hours of reperfusion. Data are expressed as the ratio of the Doppler signal intensity of the ischemic compared with the nonischemic hemisphere. Although this method does not quantify cerebral blood flow per gram of tissue, use of laser Doppler flow measurements at precisely defined anatomic landmarks serves as a means of comparing cerebral blood flows in the same animal serially over time. The surgical procedure was considered to be technically adequate if  $\geq 50\%$  reduction in relative cerebral blood flow was observed immediately after placement of the intraluminal occluding suture. These methods have been used in previous studies.<sup>7,11</sup>

**Preparation and Administration of  $^{125}\text{I}$ -Labeled Proteins and  $^{111}\text{In}$ -Labeled Murine Neutrophils**

Radioiodinated antibodies were prepared as follows: monoclonal rat anti-murine P-selectin IgG (clone RB 40.34, Pharm-

ingen Co)<sup>14</sup> and nonimmune rat IgG (Sigma Chemical Co) were radiolabeled with  $^{125}\text{I}$  by the lactoperoxidase method<sup>15</sup> using Enzymobeads (Bio-Rad). Radiolabeled PMNs were prepared in the following manner: citrated blood from wild-type mice was diluted 1:1 with NaCl (0.9%), followed by gradient centrifugation on Ficoll-Hypaque (Pharmacia). After hypotonic lysis of residual erythrocytes (20-second exposure to distilled  $\text{H}_2\text{O}$ , followed by reconstitution with 1.8% NaCl), the PMNs were suspended in PBS. Neutrophils ( $5$  to  $7.5 \times 10^6$ ) were suspended in PBS with 100  $\mu\text{Ci}$  of  $^{111}\text{In}$  oxine (Amersham Mediphsics) and subjected to gentle agitation for 15 minutes at  $37^\circ\text{C}$ . After they were washed with PBS, the PMNs were gently pelleted (450g) and resuspended in PBS to a final concentration of  $1.0 \times 10^6$  cells/mL.

**Calculation of Infarct Volumes**

After neurological examination, mice were anesthetized, and final cerebral blood flow measurements were obtained. Humane euthanasia was performed by decapitation, and brains were removed and placed in a mouse brain matrix (Activational Systems Inc) for 1-mm sectioning. Sections were immersed in 2% TTC (Sigma) in 0.9% PBS, incubated for 30 minutes at  $37^\circ\text{C}$ , and placed in 10% formalin.<sup>16</sup> Infarcted brain was visualized as an area of unstained tissue. Infarct volumes were calculated from planimetric serial sections and expressed as the percentage of infarct in the ipsilateral hemisphere. This method of calculating infarct volumes has been used previously by our group<sup>7,11</sup> and others<sup>16,17</sup> and has been correlated with the other functional indexes of stroke outcome, which are described above.

**Administration of Unlabeled Antibodies, Radiolabeled PMNs, and Radiolabeled Antibodies**

For experiments in which unlabeled antibodies were administered, one of two different antibody types was used, either a blocking monoclonal rat anti-murine P-selectin IgG (clone RB 40.34, Pharmingen Co)<sup>14,18,19</sup> or nonimmune rat IgG (Sigma). Antibodies were prepared as 30  $\mu\text{g}$  in 0.2 mL PBS containing 0.1% BSA, which was then administered into the penile vein 10 minutes before MCAO. In separate experiments, radiolabeled antibodies (0.15 mL,  $\approx 2.6 \times 10^5$  cpm/ $\mu\text{L}$ ) were injected intravenously 10 minutes before MCAO. In a third set of experiments, radiolabeled PMNs were administered intravenously 10 minutes before MCAO as a 100- $\mu\text{L}$  injection (radiolabeled PMNs were admixed with physiological saline to a total volume of 0.15 mL,  $\approx 3 \times 10^6$  cpm/ $\mu\text{L}$ ). For experiments in which unlabeled antibodies were administered, the times at which measurements were made are indicated in the text, using the methods described above to determine cerebral blood flow, infarction volumes, and mortality. For those experiments in which either radiolabeled antibodies or radiolabeled PMNs were administered, mice were killed at the indicated time points, and brains were immediately removed and divided into ipsilateral (posts ischemic) and contralateral hemispheres. Deposition of radiolabeled antibodies or neutrophils was measured and expressed as ipsilateral/contralateral counts per minute.

**Immunohistochemistry**

Brains were removed at 1 hour after reperfusion, fixed in 10% formalin, paraffin-embedded, and sectioned for immunohistochemistry. Sections were stained with an affinity-purified polyclonal rabbit anti-human P-selectin antibody (1:25 dilution, Pharmingen), and sites of primary antibody binding were visualized using a biotin-conjugated goat anti-rabbit IgG (1:20) detected with Extravidin peroxidase (Sigma).

**Data Analysis**

Cerebral blood flow, infarct volume, and  $^{111}\text{In}$ -PMN deposition were compared using Student's *t* test for unpaired variables. Two-way ANOVA was performed to test for significant differences between baseline and final (30-minute) antibody

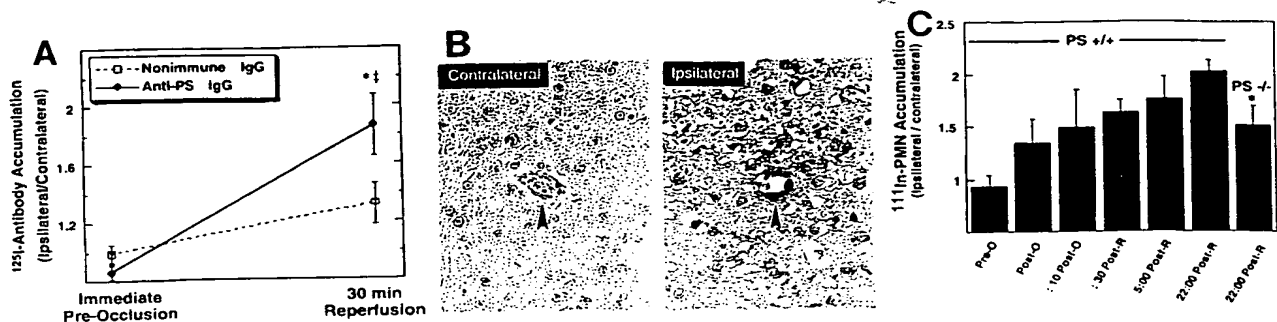
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**FIG 1.** P-selectin expression and neutrophil (PMN) accumulation after MCAO in mice. **A**, P-Selectin expression after MCAO and reperfusion. Relative expression of P-selectin antigen in the ipsilateral cerebral hemisphere after MCAO was demonstrated using either a  $^{125}\text{I}$ -labeled rat monoclonal anti-P-selectin (anti-PS) IgG or a  $^{125}\text{I}$ -labeled nonimmune rat IgG to control for nonspecific extravasation. Values are expressed as ipsilateral/contralateral counts per minute ( $n=6$  for each group, except for 30-minute control [ $n=4$ ];  $\dagger P<.001$ , 30-minute reperfusion vs immediate preocclusion;  $*P<.025$ , change in P-selectin accumulation vs change in control IgG accumulation). **B**, Immunohistochemical localization of P-selectin expression in a section of brain from a mouse subjected to 45 minutes of MCAO followed by 1 hour of reperfusion. Ipsilateral and contralateral cerebral cortical sections are shown from the same mouse. Arrows point to a cerebral microvessel, with dark brown color representing P-selectin expression at the endothelial cell surface. **C**, Time course of PMN accumulation after focal cerebral ischemia and reperfusion in the mouse. For these experiments,  $\approx 3.3 \times 10^5$   $^{111}\text{In}$ -labeled PMNs were injected intravenously into PS +/+ mice 15 minutes before MCAO.  $^{111}\text{In}$ -PMN accumulation was measured immediately after the animals were killed as the ratio of ipsilateral/contralateral counts per minute under the following experimental conditions: before MCAO (Pre-O,  $n=4$ ), immediately after MCAO (Post-O,  $n=6$ ), and 10 minutes after MCAO but still before reperfusion (10 Post-O,  $n=6$ ). To establish the effect of reperfusion on PMN accumulation, reperfusion was initiated after 45 minutes of ischemia. PMN accumulation was measured after 30 minutes (30 Post-R,  $n=6$ ), 300 minutes (5:00 Post-R,  $n=3$ ), and 22 hours (22:00 Post-R,  $n=8$ ) of reperfusion. Under identical conditions, PMN accumulation was measured in PS -/- mice after 45 minutes of ischemia and 22 hours of reperfusion ( $n=7$ ;  $*P<.05$  vs 45-minute MCAO/22-hour reperfusion in PS +/+ mice).

deposition between the two groups (experimental versus sham). Student's *t* test for unpaired variables was performed to evaluate within-group differences (baseline versus the 30-minute time point). Survival differences between groups was tested using contingency analysis with the  $\chi^2$  statistic. Values are expressed as mean  $\pm$  SEM, with a value of  $P<.05$  considered statistically significant.

## Results

### P-Selectin Expression in Murine Stroke

Because P-selectin mediates the initial phase of leukocyte adhesion to activated endothelial cells,<sup>20</sup> we examined early cerebral P-selectin expression in a murine model of reperfused stroke. Mice given a  $^{125}\text{I}$ -labeled rat monoclonal anti-murine P-selectin IgG before surgery demonstrated a 216% increase in accumulation of the antibody at 30 minutes of reperfusion compared with sham-operated mice ( $P<.001$ , Fig 1A). To demonstrate that this degree of antibody deposition in the reperfused hemisphere was due to P-selectin expression rather than nonspecific accumulation, a comparison was made with identically treated animals given a  $^{125}\text{I}$ -labeled rat nonimmune IgG. These experiments demonstrated that there was significantly greater accumulation of the anti-P-selectin IgG than the nonimmune IgG ( $P<.025$ , Fig 1A), suggesting that P-selectin is expressed in the brain within 30 minutes of reperfusion. Examination of sections of brain tissue immunostained for P-selectin reveal that P-selectin expression is primarily localized to the microvascular endothelial cells in the ipsilateral cerebral cortex (Fig 1B).

### Neutrophil Accumulation in Murine Stroke

To delineate the time course over which PMN influx occurs after stroke,  $^{111}\text{In}$ -labeled PMN accumulation was measured in PS +/+ mice before MCAO, immediately after and 10 minutes after MCAO, and at 30 minutes,

300 minutes, and 22 hours of reperfusion. In PS +/+ mice, accumulation of PMNs begins early after the initiation of focal ischemia and continues throughout the period of reperfusion (Fig 1C). To establish the role for P-selectin in this postischemic neutrophil accumulation, experiments were performed using mice that were homozygous null for the P-selectin gene (PS -/-). PS -/- mice showed significantly reduced PMN accumulation after MCAO and reperfusion (Fig 1B).

### Role of P-Selectin in Cerebrovascular No-Reflow Phenomenon

To determine whether the reduction in PMN accumulation in PS -/- mice resulted in improved cerebral blood flow after the reestablishment of flow, serial measurements of relative cerebral blood flow were obtained by laser Doppler in both PS +/+ and PS -/- mice. Before the initiation of ischemia (Fig 2, point a), relative cerebral blood flows were nearly identical between groups. MCAO (Fig 2, point b) was associated with a nearly identical drop in cerebral blood flow in both groups. Immediately before withdrawal of the intraluminal occluding suture at 45 minutes of ischemia (Fig 2, point c), cerebral blood flows had risen slightly, although they remained significantly depressed compared with baseline flows. Immediately after withdrawal of the occluding suture to initiate reperfusion (Fig 2, point d), cerebral blood flows in both groups increased to a comparable degree ( $\approx 60\%$  of baseline in the PS -/- and PS +/+ mice). The immediate failure of the postreperfusion cerebral blood flows to reach preocclusion levels is characteristic of the cerebrovascular no-reflow phenomenon,<sup>21</sup> with the subsequent decline in postreperfusion cerebral blood flows representing delayed postischemic cerebral hypoperfusion.<sup>22</sup> By 30 minutes of reperfusion (Fig 2, point e), the cerebral blood flows between the two groups of animals had diverged,

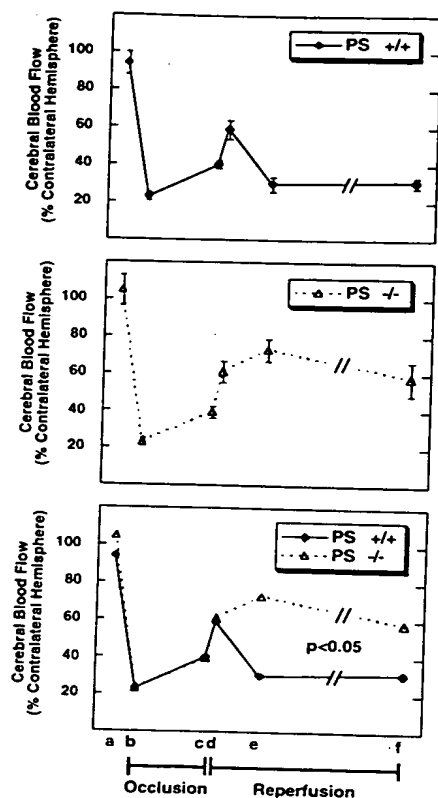


Fig 2. Role of P-selectin in the cerebrovascular no-reflow phenomenon. Cerebral blood flow was measured in PS +/+ (top) and PS -/- (middle) mice using a laser Doppler flow probe and expressed as the percentage of contralateral (nonischemic) hemispheric blood flow ( $\pm$ SEM). Blood flow was measured at the following time points: a, before MCAO (PS +/+, n=16; PS -/+, n=23); b, immediately after MCAO (PS +/+, n=42; PS -/+, n=40); c, 10 minutes after MCAO but still before reperfusion (PS +/+, n=36; PS -/+, n=34); d, immediately after reperfusion (PS +/+, n=36; PS -/+, n=34); e, 30 minutes after reperfusion (PS +/+, n=8; PS -/+, n=5); and f, 22 hours after reperfusion (PS +/+, n=15; PS -/+, n=5). The bottom panel represents an overlay of the top two panels, with error bars omitted for clarity.

with PS -/- animals demonstrating significantly greater relative cerebral blood flows than the PS +/+ control animals ( $P<0.05$ ) (Fig 2, point f). This divergence reflected significant differences in delayed postischemic cerebral hypoperfusion and persisted for the 22-hour observation period.

### Stroke Outcome

The functional significance of P-selectin expression was tested by comparing indexes of stroke outcome in PS -/- mice with those in PS +/+ control mice. PS -/- mice were significantly protected from the effects of focal cerebral ischemia and reperfusion, according to the 77% reduction in infarct volume ( $P<0.01$ ) in PS -/- mice compared with P-selectin +/+ control mice (Fig 3A). This reduction in infarct volume was accompanied by increased survival in the PS -/- mice ( $P<0.05$ , Fig 3B).

### Effect of P-Selectin Blockade

After the functional role of P-selectin expression in stroke was observed using deletionally mutant mice,

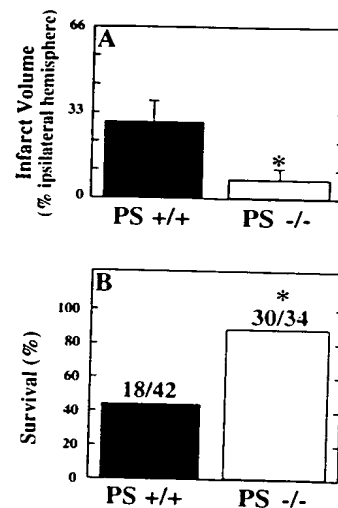


Fig 3. Effect of the P-selectin gene on stroke outcomes. MCAO was performed for 45 minutes, followed by 22 hours of reperfusion in PS +/+ (n=10) or PS -/- (n=7) mice. Effect of P-selectin on infarct volume, as evidenced by 2% TTC staining and calculated as percentage of ipsilateral hemisphere, is shown in panel A; effect of P-selectin on percent survival immediately before removal of the brain is shown in panel B. Mean  $\pm$  SEM values are indicated, with the numbers of animals from which the percentage survival was calculated indicated above the survival bars (\* $P<0.05$ ).

experiments were performed to determine whether pharmacological blockade of P-selectin could improve stroke outcome in PS +/+ mice. A strategy of administering a functionally blocking monoclonal rat anti-mouse P-selectin antibody (clone RB 40.34<sup>14,18,19</sup>) or nonimmune control rat IgG immediately before surgery was used, and mice receiving the blocking antibody immediately before MCAO were observed to have improved postreperfusion cerebral blood flows by 30 minutes, as well as reduced cerebral infarction volumes and a trend toward reduced mortality compared with control mice (Fig 4, leftmost six bars). To increase the potential clinical relevance of a strategy of P-selectin blockade as a new treatment for stroke, additional experiments were performed in which either the control or the blocking antibody was given after intraluminal occlusion of the middle cerebral artery (because most patients present after the onset of stroke). In these studies, a significant reduction in infarct volumes was observed along with a trend toward improved cerebral blood flow (Fig 4, rightmost six bars).

### Discussion

Despite substantial progress in recent years in the primary prevention of stroke,<sup>1</sup> therapeutic options to treat evolving stroke remain extremely limited. Although the recent publication of two landmark trials last fall demonstrating reduced morbidity after treatment of ischemic stroke with rt-PA<sup>2,3</sup> was thought to usher in a new era of thrombolytic therapy in the treatment of stroke,<sup>4</sup> enthusiasm has been tempered somewhat by the hemorrhagic transformation and increased mortality noted in patients with ischemic stroke treated with streptokinase.<sup>5</sup> These divergent trials make it more critical than ever that new safe therapies be developed to

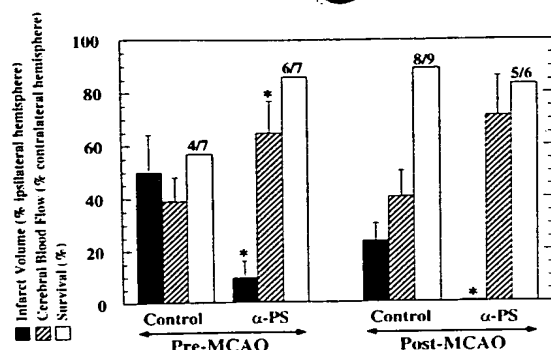


FIG 4. Effect of P-selectin blockade on stroke outcomes. PS +/+ mice were given either a blocking rat anti-mouse anti-P-selectin ( $\alpha$ -PS) IgG (clone RB 40.34, 30  $\mu$ g/mouse) or a similar dose of nonimmune rat IgG immediately before MCAO (Pre-MCAO,  $n=7$  for each group) or after occlusion of the middle cerebral artery (Post-MCAO,  $n=9$  for the control antibody and  $n=6$  for the functionally blocking  $\alpha$ -PS antibody). In both cases, the intraluminal occluding suture was withdrawn after a 45-minute ischemic period to simulate clinical reperfusion. After 22 hours of reperfusion, infarct volumes (solid bars), relative cerebral blood flow at 30 minutes after reperfusion (hatched bars), and survival (open bars) are shown. Mean  $\pm$  SEM values are indicated, with the numbers of animals from which the percentage survival was calculated indicated above the survival bars. \* $P<.05$  vs control antibody.

treat evolving stroke. Although restoration of blood flow to postischemic brain affords new opportunities for early therapeutic intervention, reperfusion is a double-edged sword. Given the cytotoxic potential of neutrophils,<sup>23</sup> it is not surprising that neutrophil influx into postischemic brain tissue can lead to further damage and worsen the outcome after experimental stroke.<sup>7,24-27</sup> Using a murine model of focal cerebral ischemia and reperfusion, we have recently identified an important contributory role for the cell adhesion molecule ICAM-1 in neutrophil accumulation at 22 hours after stroke.<sup>7</sup> However, augmented cerebrovascular endothelial ICAM-1 expression requires *de novo* transcriptional and translational events, which require time. In contrast, P-selectin, a membrane-spanning glycoprotein that mediates the earliest phases of neutrophil adhesion, may be mobilized from preformed storage pools to be rapidly expressed at the ischemic endothelial cell surface.<sup>8,28</sup> Since the clinical trials of thrombolytic therapy for stroke demonstrate a narrow time window for potential benefit (within the first several hours of stroke onset),<sup>2,3,5</sup> strategies designed to interfere with the earliest phases of PMN adhesion might be of theoretical benefit in human stroke. These trials should result in greater numbers of patients presenting for earlier therapeutic intervention, increasing the need to address the issue of reperfusion injury in medically revascularized territories. In addition, they underscore the pressing need to understand the contributions of individual adhesion molecules to the pathogenesis of stroke.

Given the considerable body of literature describing the role of P-selectin in other models of ischemia and reperfusion,<sup>8,29-32</sup> surprisingly little is known about the role of P-selectin in stroke. Knowledge of the specific role of P-selectin in the cerebral vasculature is important because adhesion molecule requirements vary between

vascular beds and conditions under study. For instance, in a model of intestinal transplantation,<sup>33</sup> anti-P-selectin antibodies did not reduce reperfusion injury, whereas anti-CD11/CD18 antibodies did. Although P-selectin blockade was ineffective at reducing PMN adhesion and albumin leakage in a rat mesenteric ischemia and reperfusion model, ICAM-1 blockade was effective.<sup>34</sup> In a rat hindlimb ischemia/reperfusion model, the selectin requirements for PMN adhesion differed between the pulmonary and crural muscle vascular beds.<sup>31</sup>

To our knowledge, the only published study describing increased P-selectin expression in the ischemic brain is a histopathological description of primate stroke, in which P-selectin expression was increased in the lenticulostriate microvasculature.<sup>10</sup> The present studies were undertaken to study whether P-selectin expression contributes to postischemic cerebral neutrophil accumulation, the no-reflow phenomenon, and tissue injury in a murine model of reperfusion stroke. Using a recently established model of focal cerebral ischemia and reperfusion in mice,<sup>11</sup> P-selectin expression was demonstrated by increased endothelial immunostaining and increased deposition of radiolabeled antibody in the ischemic territory. In the latter technique, antibody deposition into the ischemic hemisphere was normalized to that in the nonischemic hemisphere in each animal, not only to minimize potential variations in injection volume or volume of distribution but also to enable comparison between animals given different antibodies. Because disruption of the endothelial barrier function in the ischemic cortex may augment nonselective antibody deposition, similar experiments were performed with a control rat IgG. These data show that the antibody that binds to P-selectin is deposited at an accelerated rate compared with the control antibody, suggesting that local P-selectin expression is augmented in the reperfusion tissue. These data in the murine model parallel the data reported in a baboon model of stroke,<sup>10</sup> in which P-selectin expression was increased within 1 hour after the ischemic event.

The role of P-selectin expression in recruiting PMNs to the postischemic zone was demonstrated using a strategy in which accumulation of <sup>111</sup>In-labeled PMNs was measured. Although we have previously reported that by 22 hours PMN accumulation is elevated in the ischemic hemisphere,<sup>7</sup> the present time-course data demonstrate that PMN accumulation begins shortly after the onset of ischemia. Failure to express the P-selectin gene was associated with reduced PMN accumulation, suggesting the participation of P-selectin in postischemic cerebral PMN recruitment. However, the P-selectin-null animals did demonstrate a modest (albeit less than control) neutrophil accumulation by 22 hours. These data indicate that P-selectin is not the exclusive effector mechanism responsible for postischemic cerebral PMN recruitment and are consistent with our previous data showing that ICAM-1 also participates in postischemic PMN adhesion.<sup>7</sup> Furthermore, these data are not unlike data in which intra-abdominal instillation of thioglycolate in P-selectin-deficient mice caused delayed (but not absent) PMN recruitment.<sup>9</sup>

Because of the critical need to identify reasons for failed reperfusion, the present studies examined the role of P-selectin in delayed postischemic cerebral hypoperfusion,<sup>21,22</sup> the phenomenon wherein blood flow declines

during reperfusion, despite restoration of adequate perfusion pressures. In cardiac models of ischemia, the no-reflow phenomenon worsens as time elapses after reperfusion,<sup>35</sup> suggesting an important role for recruited effector mechanisms, such as progressive microcirculatory thrombosis, vasomotor dysfunction, and PMN recruitment. Both P-selectin- and ICAM-1-dependent adherence reactions<sup>36</sup> and PMN capillary plugging<sup>37</sup> have been shown in other models to participate in the postischemic no-reflow phenomenon. In the brain, PMNs have been implicated in the postischemic cerebral no-reflow phenomenon,<sup>38,39</sup> but the role of P-selectin had not been previously elucidated.

The present study uses a relatively noninvasive technique (laser Doppler) to obtain serial measurements of relative cerebral blood flow in order to establish the existence, time course, and P-selectin dependence of the postischemic cerebrovascular no-reflow phenomenon. In order to demonstrate that the threading procedure itself was not the cause of vascular damage and subsequent cerebral infarction, experiments involving sham ischemia were performed ( $n=10$ ) in which a nylon suture was threaded into the internal carotid artery for a 45-minute nonoccluding period. In these experiments, the threading was shown to be nonocclusive because there was no decline in perfusion by laser Doppler during the 45-minute period. When brains were then collected and stained with TTC at 24 hours, none showed evidence of cerebral infarction. Therefore, we can conclude that the threading procedure per se does not provoke sufficient damage to affect our major outcome variables. When relative cerebral blood flow was examined after frank MCAO in experimental animals, we observed that P-selectin-null and control animals were subjected to virtually identical degrees of ischemia (there was an initial  $\approx 4.5$ -fold drop in relative cerebral blood flow after MCAO in both). However, there was a slight increase in relative cerebral blood flow in the first 10 minutes after occlusion, even though the occluding suture remained in place. This is an empiric observation that we have consistently made, for which there are likely to be several possible explanations. There is likely to be some degree of collateral flow that opens up in the ischemic territory. Another tenable explanation is that there may be an element of initial vasospasm in the region of the occluding catheter tip that modestly resolves within several minutes. Although both of these explanations are possible, because of the small size of the murine vasculature, we cannot identify the mechanism with certainty in our model. Nevertheless, because we observed the same degree of flow recruitment in both control and experimental animals, these data do not alter our main conclusion, that P-selectin is an important mediator of cerebral tissue injury in reperfused stroke.

After removal of the intraluminal occluding suture, instantaneous recovery of blood flow was the same in both the P-selectin  $+/+$  and  $-/-$  animals. The fact that flow levels never returned to baseline (nor was there an overshoot, as might be seen with reactive hyperemia) may be due to the severity and duration of the ischemic period, which is likely to recruit other mechanisms of the postischemic cerebrovascular no-reflow phenomenon, such as thrombosis or neutrophil recruitment caused by non-selectin-dependent mechanisms. When even later time points are examined (such as 30 minutes to 22

hours after removal of the occluding suture), it is interesting to note that there is a slight decline in cerebral blood flow in the PS  $-/-$  animals. This late (albeit limited) decline in cerebral blood flow by 22 hours is consistent with the modest PMN recruitment observed in the PS  $-/-$  animals over the same period, again suggesting the recruitment of other flow-limiting effector mechanisms (such as ICAM-1) in the PS  $-/-$  animals.

The functional effects of P-selectin expression are clear from the present set of studies: animals that fail to express the P-selectin gene (or PS  $+/+$  animals treated with a functionally blocking anti-P-selectin antibody) exhibit smaller infarcts and improved survival compared with control animals. When these data are considered along with previously published data demonstrating a deleterious role for ICAM-1 expression in stroke,<sup>7</sup> it becomes increasingly apparent that there are multiple means for recruiting PMNs to the postischemic cerebral cortex and that blockade of each represents a potential strategy to improve stroke outcome in humans. Given our current recognition of the importance of timely reperfusion in halting the advancing wave front of neuronal death after stroke, interfering with PMN adhesion at its earliest stages appears to be an attractive option for reducing morbidity and mortality. In fact, anti-adhesion molecule strategies may not only be beneficial in their own right (ie, including patients ineligible for thrombolysis) but may extend the window of opportunity for thrombolytic intervention.<sup>40</sup> The present set of studies contributes to our understanding of pathophysiological mechanisms operative in reperfused stroke. These studies suggest the need for clinical trials of therapies for evolving stroke that optimize the reperfusion milieu to reduce PMN accumulation.

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